REMARKS

The present Amendment is in response to the Examiner's Office Action mailed August 28, 2003. Claims 1-4, 7-9, 12, 13, 15, 18, 20, and 22 are pending in the application. Claims 5, 6, 10, 11, 14-19, and 21-29 are cancelled. Claims 30 and 31 are withdrawn. By this Amendment, claims 1, 3, 13 and 20 are amended, and claims 15, 18 and 22 are cancelled.

By this amendment, independent claims 1 and 3 have been amended to more clearly define the claimed invention. Support for the amended method claim can be found in the specification at pages 33, 42 and 49 *inter alia*. The subject matter of previously dependent claims 15, 18 and 22 is now included as a Markush group in amended claim 13 and claims 15, 18 and 22 are cancelled. The dependency of claim 20 has has been changed to amended, independent claim 13. No new matter has been added. Amendment and cancellation of certain claims is not to be construed as a dedication to the public of any of the subject matter of the claims as previously presented.

Claims 1-4, 7-9, 12, 13 and 20 are currently under consideration. Reconsideration of the application is respectfully requested in view of the above amendments to the claims and the following remarks. For the Examiner's convenience, Applicants' remarks are presented in the order in which they were raised in the Office Action.

Allowable claims

(i)   <u>Applicants appreciate the determination by the Examiner that claims 13, 15, 18, 20 and 22 contain allowable subject matter if redrafted in independent form.</u>

In response, Applicants have rewritten claim 13 in independent form having incorporated the language of independent claim 1. Applicants have listed the subject matter of previously dependent claims 15, 18 and 22 as a Markush group in claim 13 and cancelled the corresponding claims 15, 18 and 22. The dependency of claim 20 has has been changed from the now cancelled claim 18 to amended, independent claim 13. No new matter is added. Applicants believe that, as amended, claims 13 and 20 are now allowable.

Applicants also express their appreciation for withdrawal of the prior rejection under 35 USC §112.

Rejection Under 35 U.S.C. § 102 (e)

(ii)   The Examiner has maintained the rejection of claims 3-4 under 35 U.S.C. 102(e) as being anticipated by Curtiss, III et al (US Pat. 6, 383, 496) for the reasons of record.

In general, Applicants note that while there are references that teach the alteration of Dam activity, inventors of the present invention are the first to show novel uses of selected pathogenic bacteria wherein such alteration has resulted in attenuation of the bacteria to virulence levels that are within safety standards and novel uses of such bacteria in engendering immunogenic compositions.

The Application describes the meaning of "immunogenic compositions" as compositions that are able to elicit "a specific antibody (B cell) or cellular (T cell) immune response or both." (Specification, page 49, para 184). "Attenuated bacteria" are defined as bacteria with virulence reduced to a "suitable and acceptable safety level" (Specification, page 33, para 129). The degree of reduction in virulence depends on the recipient as well as regulatory agencies. (Specification, page 33, para 130). Further, the present application teaches that bacteria are "screened for virulence." (Specification, page 42, para 162).

In response, Applicants amend claim 3 to specify that the attenuated bacteria contain an "alteration [of Dam activity] ... in a manner which renders the live bacteria attenuated such that virulence of the attenuated bacteria is reduced to an acceptable safety level."

The Examiner cites Curtiss III to show that it inherently discloses bacteria with altered Dam activity wherein the bacteria contains other plasmids. However, the priority Curtis reference (U.S. App. Ser. No. 08/970,789, issued as US Pat. No 6,024,961) does not teach or suggest an "attenuated" live bacteria with an altered Dam gene such that virulence of the attenuated bacteria is reduced to an acceptable safety level."

Since Curtiss III does not teach each and every element of independent claim 3 and claim 4 depends from claim 3, Applicants respectfully request withdrawal of this ground for rejection

Rejections Under 35 U.S.C. § 102 (b)

(iii)   <u>Claims 1-2, 3-4, 7-9, and 12 are newly rejected under 35 U.S.C. 102(b) as being anticipated by Collier et al (US Pat. 5,451,519).</u>

Collier is cited for disclosing an attenuated live bacteria wherein the E. coli strain has an altered dam activity. Applicants respectfully traverse.

Independent claim 1 is directed to a "immunogenic composition" wherein the "with the alteration [of the Dam activity] being in a manner which renders the live bacteria attenuated." Collier is directed to methods for cloning genes for restriction enzymes by altering dam activity in host bacterial cells. While Collier may disclose bacteria where dam activity is altered,Collier does not teach or suggest either an immunogenic composition nor an "attenuated" live bacteria. The only reference to a "immune" system in Collier is in col. 1, lines 30-39, where methylation pattern is described as a simple "immune" system for bacteria for comparative purposes. It is well-known that bacteria do not have an immune system.

As discussed above, the Application clearly describes the meaning of "immunogenic" as compositions that are able to elicit "a specific antibody (B cell) or cellular (T cell) immune response or both." (Specification, page 49, para 184). "Attenuated bacteria" are defined as bacteria with virulence reduced to a "suitable and acceptable safety level" (Specification, page 33, para 129). The degree of reduction in virulence depends on the recipient as well as regulatory agencies. (Specification, page 33, para 130). Further, the present application teaches that bacteria are "screened for virulence." (Specification, page 42, para 162).

Solely for the purppose of clarifying the claimed invention Applicant amend independent claims 1 and 3 to specify attenuated bacteria wherein "virulence of the attenuated bacteria is reduced to an acceptable safety level."

Since Collier does not teach a bacteria with a dam alteration that has its virulence reduced to a suitable safety level as defined in the Specification it does not teach "an attenuated form of bacteria" as specified in claim 1. Therefore, Applicants respectfully request that these grounds for the rejection of claim 1 under 35 USC 102(b) over Collier be withdrawn.

With respect to independent claim 3, the Examiner cites bacteria with a first heterologous sequence expressing T3 SAM modifying enzymes and a second heterologous sequence expressing Bam HII methyl transferase. Neither is disclosed as an antigen that forms an immunogenic composition. Antigen is defined as a substance "that is recognized and bound specifically by an antibody or by a T cell antigen receptor." (Specification, page 33, para 130). Collier does not teach that either T3 SAM modifying enzymes or BamHII methyl transferase is an antigen. As discussed above, Collier also does not teach "attenuated" bacteria, i.e., bacteria with dam activity altered to a sufficient level to display an acceptable level of reduced virulence.

The Examiner also cites to cols. 20 and 21 of Collier for teaching an "acceptable excipient." Applicants note that Collier (at 20:1-6; 20:54-56 and 20:64 - 21:2) teaches broth for growing bacteria containg multiple antibiotics. The excipients are certainly not "pharmaceutically acceptable" as described in page 39, para 152 of the Specification because a pharmaceutically acceptable excipient must be suitable for administration to live birds, animals and humans.

Since Collier does not teach attenuated bacteria wherein "virulence of the attenuated bacteria is reduced to an acceptable safety level" as specified in amended, independent claims 1 and 3, it does not teach each and every element of independent claims 1 and 3 and claims 2, 4, 7-9 and 12 are dependent claims thereof, Applicants respectfully request withdrawal of this ground for rejection.

(iv)   Claims 1 and 7 are rejected under 35 U.S.C. 102(b) as being anticipated by Anderson et al (US Pat. 4,798,791).

Anderson is directed to methods and vectotrs for high level expression of genes in bacteria. Anderson discloses and claims bacteria (including E coli) with dam mutations and suspension of such bacteria in fermentation medium and other excipients.

Anderson does not teach or disclose bacteria which are attenuated by alteration of dam activity, immunogenic compositions or attenuated bacteria wherein "virulence of the attenuated bacteria is reduced to an acceptable safety level" as specified in amended claim 1. Anderson also does not teach or disclose excipients that are "pharmaceutically acceptable."

Since Anderson does not teach each and every element of claims 1 and 7, Applicants respectfully request withdrawal of this ground for rejection.

(v)   Claims 1 and 7 are rejected under 35 U.S.C. 102(b) as being anticipated by Berg et al (US Pat. 5,595, 736).

Berg is cited for teaching an attenuated dam altered bacteria wherein a heterologous antigen is expressed. Applicants respectfully traverse..

Berg in Examples 1D and 6, disclose transformation of a dam⁻ E. Coli with an expression plasmid. Berg does not teach or suggest that the dam⁻ E. Coli strain K12 GM48 is "attenuated" such that "virulence of the attenuated bacteria is reduced to an acceptable safety level" as specified in amended claim 1. The excipients disclosed by Berg are LB-agar plates (11:60-62) for growing bacteria and are not "pharmaceutically acceptable."

Since Berg does not teach each and every element of claims 1 and 7, Applicants respectfully request withdrawal of this ground for rejection.

(vi)   Claims 1 and 7 are rejected under 35 U.S.C. 102(a) as being anticipated by Shapiro et al (WO98/12206).

Shapiro is directed to novel DNA methyltransferase genes of the ccrM (cell cycle regulated methyltransferse) family. ccrM is not the same as Dam as they belong to separate methyltransferase groups. (*see* page 3065, col. 2, lines 10-12 of Kahng and Shapiro, J. Bact. 183(10): 3065-3075 (May 2001) and as such it does not disclose Dam altered bacteria. A copy of the article is attached for the Examiner's convenience; note that the article is authored by the inventors of the Shapiro reference)

Shapiro also does not teach or suggest alteration of Dam activity leading to attenuation of the bacteria such that "virulence of the attenuated bacteria is reduced to an acceptable safety level" as specified in amended claim 1. The excipients disclosed by Shapiro are SB-agar plates (pp. 37-38) for growing bacteria and are not "pharmaceutically acceptable."

Since Shapiro is directed to ccrM (and not Dam) and does not teach each and every element of claims 1 and 7, Applicants respectfully request withdrawal of this ground for rejection.

CONCLUSION

In light of the amendments and arguments set forth above, Applicants earnestly believe that they are entitled to a letters patent and respectfully request the Examiner to expedite prosecution of this patent application to issuance. Should the Examiner have any questions, the Examiner is encouraged to telephone the undersigned.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no.220002060724. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: December 29, 2003   Respectfully submitted,

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The CcrM DNA Methyltransferase of *Agrobacterium tumefaciens* Is Essential, and Its Activity Is Cell Cycle Regulated

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DNA methylation is now recognized as a regulator of multiple bacterial cellular processes. CcrM is a DNA adenine methyltransferase found in the alpha subdivision of the proteobacteria. Like the Dam enzyme, which is found primarily in *Escherichia coli* and other gamma proteobacteria, it does not appear to be part of a DNA restriction-modification system. The CcrM homolog of *Agrobacterium tumefaciens* was found to be essential for viability. Overexpression of CcrM is associated with significant abnormalities of cell morphology and DNA ploidy. Mapping of the transcriptional start site revealed a conserved binding motif for the global response regulator CtrA at the −35 position; this motif was footprinted by purified *Caulobacter crescentus* CtrA protein in its phosphorylated state. We have succeeded in isolating synchronized populations of *Agrobacterium* cells and analyzing their progression through the cell cycle. We demonstrate that DNA replication and cell division can be followed in an orderly manner and that flagellin expression is cyclic, consistent with our observation that motility varies during the cell cycle. Using these synchronized populations, we show that CcrM methylation of the chromosome is restricted to the late S phase of the cell cycle. Thus, within the alpha subdivision, there is a conserved cell cycle dependence and regulatory mechanism controlling *ccrM* expression.

DNA methylation, long known to exist in both eukaryotes and prokaryotes, is a widespread regulator of bacterial cellular processes. In eukaryotes, it plays important roles in transcriptional regulation and genomic imprinting and is essential for proper development in the mouse (2, 16). Among prokaryotes, it is primarily found in the context of restriction-modification systems, where it allows an organism to distinguish foreign DNA from its own. However, regulatory functions mediated by methylation have been extensively characterized for the Dam (for "DNA adenine methyltransferase") methyltransferase of *Escherichia coli*. Dam, termed an 'orphan' methyltransferase because it has no known cognate restriction enzyme, is involved in methyl-directed mismatch DNA repair as well as ensuring that multiple origins of chromosomal replication within the cell fire synchronously (23).

Dam has also been shown to influence the transcription of a growing number of genes important in the pathogenesis of bacterial diseases. The primary example of methylation-influenced transcription is that of the pyelonephritis-associated pilus, or *pap*, operon in uropathogenic *E. coli* (3, 27, 42). Methylation has now been found to also regulate phase variation of two other surface proteins: the plasmid-encoded fimbriae (Pef) of the enteric bacterium *Salmonella enterica* serovar Typhimurium, which mediate adhesion to mouse intestinal epithelium, and the nonfimbrial *E. coli* outer membrane protein Ag43 (13, 25). Furthermore, Dam has been shown to influence virulence of *S. enterica* serovar Typhimurium, where a *dam*-negative strain has severely attenuated infectivity in a mouse model and confers protection against challenge with a wild-type strain (11, 14). In a cell culture model, *dam*-negative *S.*

enterica serovar Typhimurium also exhibits defects in the invasion of epithelial cells, secretion of type III effector proteins, and cytotoxicity to M cells (11).

CcrM (for "cell cycle-regulated methyltransferase") is the best-characterized orphan methyltransferase aside from Dam. It is an essential DNA methyltransferase of the dimorphic aquatic bacterium *Caulobacter crescentus* (40). Both CcrM and Dam catalyze the transfer of a methyl group from *S*-adenosylmethionine to the N-6 position of adenine in a specific target sequence. However, they belong to separate methyltransferase groups, since their catalytic and *S*-adenosylmethionine-binding domains are organized in a different linear order (21). Their target sequences also differ: GATC for Dam and GANTC for CcrM. CcrM is widely distributed in the alpha subdivision of proteobacteria, which includes *C. crescentus, Sinorhizobium meliloti, Brucella abortus*, and *Agrobacterium tumefaciens* (40). In contrast, Dam is found primarily in the enteric bacteria and other gamma proteobacteria (32).

In *Caulobacter*, CcrM is highly regulated: its presence is restricted to a narrow window of the cell cycle by the dual mechanisms of transcriptional control and proteolysis (41, 46, 48). The *Caulobacter* CtrA response regulator, which controls multiple cellular events such as initiation of flagellar biogenesis and repression of chromosomal replication initiation, also induces transcription of *ccrM* just prior to cell division (30). Rapid proteolysis of CcrM occurs via a Lon protease-mediated pathway (46). Thus, replicating *Caulobacter* DNA is maintained in the hemimethylated state until the completion of replication, and its transition to full methylation occurs as a consequence of a burst of CcrM synthesis late in the cell cycle. Without this tight control, normal coordination between DNA replication and cell division fails to occur. If *ccrM* is constitutively transcribed throughout the cell cycle or if Lon is absent, then active CcrM protein is present throughout the cell cycle

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
E. coli		
DH5αF'	Cloning strain	Gibco-BRL
S17-1	Conjugal transfer of plasmids to A. tumefaciens	36
A. tumefaciens		
A136	Strain C58 cured of pTi plasmid	S. Long
A348	A136 containing pTiA6NC	P. Zambryski
NT1REB	Flagellum-free derivative of NT1RE (C58 derivative)	8
LS3303	A348 containing pLK308	This study
LS3304	A348 containing pLK309	This study
LS3307	A348 with pLK311 integrated 5' of spectinomycin cassette	This study
LS3308	A348 with pLK311 integrated 3' of spectinomycin cassette	This study
LS3314	A348 containing pRK290(20R)	This study
LS3315	A348 containing pJS71	This study
Plasmids		
pBluescript II SK(+)	Amp[r] cloning vector	Stratagene
pCR2.1-TOPO	Amp[r] Kan[r] cloning vector for PCR products	Invitrogen
pCRII	Amp[r] Kan[r] cloning vector for PCR products	Invitrogen
pRK290(20R)	pRK290 with pIC20R polylinker, Tet[r]	M. R. K. Alley
pJS71	Spect[r] pBBR1MCS derivative	38
pHP45	Source of Spect[r] Ω cassette	29
Agro3	Cosmid containing A. tumefaciens ccrM homolog	This study
pCS263	pCRII containing PCR of partial A. tumefaciens ccrM homolog	C. Stephens
pNPTS138	pLitmus 38-derived vector with added nptI, sacB, and RK2 oriT, Kan[r]	38
pLK300	pBluescript II SK(+) containing 2.2-kb BbsI fragment of Agro3 with A. tumefaciens ccrM homolog	This study
pLK307	pBluescript II SK(+) containing 1.5-kb ApaLI-ScaI fragment of pLK300 with ccrM and its promoter	This study
pLK308	pRK290(20R) + XbaI-XhoI fragment from pLK307 containing complete A. tumefaciens ccrM gene and its promoter	This study
pLK309	pJS71 + XbaI-XhoI fragment from pLK307 containing complete A. tumefaciens ccrM gene and its promoter	This study
pLK310	pLK300 derivative with replacement of part of ccrM by spectinomycin resistance cassette	This study
pLK311	pNPTS138 with EcoRI-SpeI fragment containing disrupted A. tumefaciens ccrM homolog from pLK310	This study

[a] Amp, ampicillin; Kan, kanamycin; Spect, spectinomycin; Tet, tetracycline.

and the chromosomal DNA is fully methylated at all times. When this occurs, cells have a filamentous morphology and an increase in their DNA content (46, 48).

DNA methyltransferases are reasonable antimicrobial drug targets. This has been highlighted by the discovery that dam-negative salmonellae are avirulent (15). Unlike Dam, CcrM has been found to be essential for viability in multiple bacteria, thus raising the possibility that inhibitors of methylation may even be bactericidal in some cases. Recent work has now implicated CcrM-mediated DNA methylation as a modulator of intracellular survival of the animal pathogen B. abortus (34). Characterizing the role of this methyltransferase in bacteria with varied ecological niches and growth cycles will allow a better understanding of its physiological importance and its potential as a drug target.

We describe here the cloning and characterization of the CcrM homolog from the plant pathogen A. tumefaciens. A. tumefaciens is the causative agent of crown-gall disease in plants, and most work on this organism has focused on the mechanisms by which it induces tumor formation. Its recently sequenced Ti plasmid carries most of the genes required for tumorigenesis, which involves the conjugal transfer of an oncogenic piece of DNA (T-DNA) into the nuclei of plant cells (47). Because of this ability to export DNA, A. tumefaciens has become instrumental in the genetic engineering of plants. Its chromosomal complement is less well studied but includes genes important for virulence. These include a locus mediating attachment to plants and a two-component signal transduction system required for tumorigenesis (7, 24).

We show here that the A. tumefaciens ccrM homolog is a chromosomally encoded gene essential for viability. Its over-expression results in a significant increase in the level of genomic DNA methylation, which correlates with aberrations of cell division and DNA content. We have mapped the transcriptional start site and found that the promoter has extensive homology to the ccrM promoters of both C. crescentus and B. abortus. Significantly, it contains a conserved binding motif for the global response regulator CtrA, which has been found in several alpha proteobacteria (1). Purified C. crescentus CtrA protein footprints this binding site in a phosphorylation-dependent manner. Finally, we show that A. tumefaciens cells can be studied as synchronous populations. Using these synchronized cells, we show that the methylation state of the A. tumefaciens chromosome varies as a function of the cell cycle. These last observations suggest that within the alpha subdivision of proteobacteria, there exists a conserved cell cycle dependence and regulatory mechanism controlling the expression of this essential DNA methyltransferase.

MATERIALS AND METHODS

Bacterial growth conditions and media. Strains and plasmids used are listed in Table 1. E. coli was grown at 37°C in Luria-Bertani (LB) medium containing 10 g of NaCl/liter. A. tumefaciens was grown at 28°C in YEB (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 2 mM MgSO₄), LB containing 5 g of NaCl/liter, or AB minimal medium (6) supplemented with 0.5% glucose under normal conditions or with 5% sucrose when used for sacB counterselection (see below). For solid media, 15 g of agar was added per liter. Antibiotics were used at the following concentrations: nalidixic acid, 20 µg/ml; kanamycin, 25 µg/ml for A. tumefaciens and 50 µg/ml for E. coli; spectinomycin, 200 µg/ml for A. tume-

A.

━━━━━━ catalytic domain   ━━━━━━━➤ SAM-binding
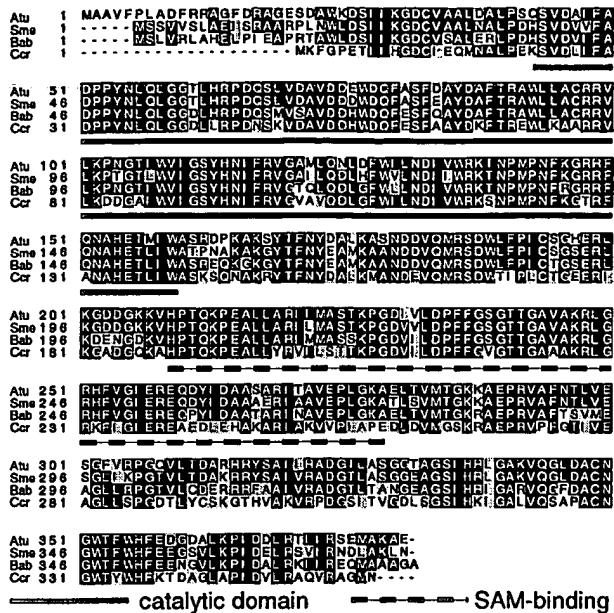
B.
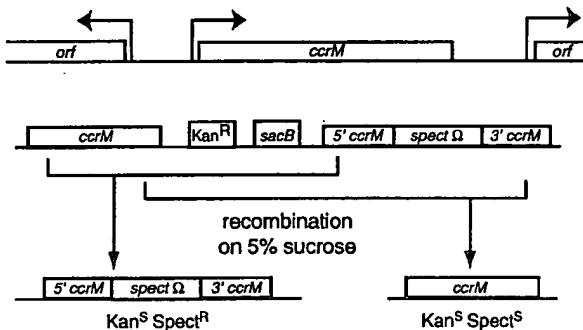
C.

Chromosomal genotype of isolates after excision of pLK311 (% of Kan^S isolates)

Strain	Plasmid	wt *ccrM* Kan^{S}Spect^S	disrupted *ccrM* Kan^{S}Spect^R
LS3307	none	100	0
LS3308		100	0
LS3307	vector alone	100	0
LS3308		100	0
LS3307	vector + *ccrM*	39	61
LS3308		69	31

FIG. 1. The *A. tumefaciens ccrM* homolog is essential for viability. (A) Homology diagram of the *A. tumefaciens* (Atu) CcrM homolog in relation to the other homologs from *S. meliloti* (Sme), *B. abortus* (Bab), and *C. crescentus* (Ccr). Black boxes denote identical amino acids and shaded boxes denote similar amino acids. The catalytic and *S*-adenosylmethionine-binding domains are marked. (B) (Top) Diagram of the *ccrM* locus. (Bottom) Diagram of the locus following integration of the

faciens and 50 μg/ml for *E. coli*; tetracycline, 12 μg/ml; and rifampin, 100 μg/ml. Plasmids were introduced into *A. tumefaciens* either by electroporation or by mating with *E. coli* S17-1 as a donor strain (6).

Cloning the full-length *A. tumefaciens ccrM* gene. Degenerate primers were designed based on regions of homology common to the *C. crescentus* and *Haemophilus influenzae* methyltransferases (45). Primer 1 (5′-AT[C/T]TT[C/T]G C[CGT]GA[C/T]CC[C/G/T]TA-3′) and primer 2 (5′-TC[A/G]TT[G/C]A[A/G] [A/G]ATCCA[A/G]AA-3′) were used in PCR with genomic DNA from *A. tumefaciens*. The PCR fragment was cloned into the pCRII vector (Invitrogen) as pCS263, and the insert was used to screen an *A. tumefaciens* A136 genomic library (7). Two clones were isolated and characterized. A 2.2-kb *Bbs*I fragment containing the entire *A. tumefaciens ccrM* homolog was isolated from the cosmid Agro3 and cloned into the *Sma*I site of pBluescript II SK(+) (Stratagene) to generate pLK300. The gene was sequenced using either a Thermosequenase kit (Amersham) or automated sequencing (Applied Biosystems 373A; Bio101, La Jolla, Calif.). Sequence analysis and alignment were performed with the Wisconsin Package, version 10.1 (Genetics Computer Group, Madison, Wis.). Alignment was performed with ClustalW, followed by shading of the alignment with Boxshade.

Construction of the *A. tumefaciens ccrM* null mutant. To determine whether *ccrM* is essential in *A. tumefaciens*, we replaced the nucleotide sequence encoding the catalytic domain of the methyltransferase with a spectinomycin resistance cassette (Fig. 1B). The spectinomycin resistance cassette from pHP45 (29) was cut out with *Sma*I and ligated into a blunt-ended *Pfl*MI-*Stu*I digest of pLK300 to give pLK310. An *Eco*RI-*Spe*I fragment of pLK310 with the gene and cassette was then cloned into a suicide vector (pNPTS138) bearing a kanamycin resistance marker as well as the *sacB* gene of *Bacillus subtilis*, yielding pLK311. The levansucrase encoded by *sacB* produces a toxic metabolite when cells are grown on sucrose, allowing eventual counterselection against the presence of the plasmid. Only kanamycin-sensitive colonies were scored, because some sucrose-resistant colonies had inactivation of the *sacB* gene and therefore had not truly excised pLK311. For complementation, the intact gene and its upstream region were cloned into the low-copy-number vector pRK290(20R) to give pLK308. Tetracycline was used to maintain the control and complementing plasmids.

Microscopy and flow cytometry. For analysis of mixed cultures, *A. tumefaciens* cells were grown to exponential phase ($A_{600} \sim 0.5$) and then treated with 1/100 volume of fix solution (12.5% formaldehyde, 150 mM sodium phosphate [pH 7.5]) for 15 min at room temperature. For synchronized cell populations, 1/5 volume of fix solution was used to ensure immediate fixation. Cells were then washed twice in growth medium and stored at 4°C. For flow cytometry, cold ethanol was added to a final concentration of 70%. Without light formaldehyde fixation, the cells tended to lyse when ethanol was added. Samples were then visually examined under the light microscope to ensure preservation of the morphology as seen in the starting culture. For flow cytometry, cells were gently recentrifuged and resuspended in TMS buffer (10 mM Tris-HCl [pH 7.2], 1.5 mM MgCl₂, 150 mM NaCl) containing 10 μg of chromomycin A₃/ml (44). The DNA content of 10,000 cells from each sample was analyzed on a Becton Dickinson FACStar instrument with excitation at 458 nm and measurement of fluorescence at 495 nm. The data were collected and analyzed using FlowJo software (Tree Star Inc., San Carlos, Calif.).

For microscopy, the fixed, washed cells were placed on a 1% agarose pad and the coverslips sealed with melted Valap (1:1:1; vaseline-lanolin-paraffin wax). Samples were photographed using a Nikon E800 microscope with a 100× DIC

plasmid pLK311, yielding tandem copies of the gene, one of which is disrupted by a spectinomycin resistance cassette. The resulting strain is resistant to both kanamycin and spectinomycin. Growth on 5% sucrose with excision of the vector sequences leads to two possible outcomes. If excision occurs on the opposite side of the Ω cassette from the original integration event, a disrupted copy of the gene will remain on the chromosome and the resulting strain will be spectinomycin resistant but kanamycin sensitive. If excision occurs on the same side as the original integration event, then the strain will be wild type and sensitive to both spectinomycin and kanamycin. (C) Only the presence of *ccrM* in *trans* allows viable colonies when the chromosomal copy of *ccrM* is disrupted. Strain LS3307 carries the pLK311 vector recombined on the 5′ side of the spectinomycin resistance cassette, and LS3308 carries pLK311 recombined on the 3′ side of the cassette. Results are shown for both strains with no plasmid, vector alone [pRK290(20R)], or vector plus *ccrM* (pLK308). A minimum of 100 isolates were scored for each experiment.

(differential interference contrast) objective and either a Hamamatsu C2400 camera controlled by Scion Image software (Scion Corp., Frederick, Md.) or a MicroMAX cooled charge-coupled device camera (Princeton Instruments, Trenton, N.J.) controlled by METAMORPH 3.6A (Universal Imaging, Media, Pa.).

Determination of the methylation state of the chromosome in *A. tumefaciens* and assessment of the Ti plasmid and chromosomal DNA content. The DNA methylation state of an overlapping *Hinc*II-*Hinf*I site located in the *att* locus was assessed in the *A. tumefaciens* strains A348, LS3314, LS3315, LS3303, and LS3304. Genomic DNA was isolated (PureGene; Gentra Systems) at similar times in the exponential growth phase of the strains, and equal amounts were digested for each lane of a gel. Southern blots of the *Hinc*II-digested genomic DNA were probed with a 500-bp PCR product generated by using the primers Agro att forward (5'-GTAGAGGTTGAGAAGTGTAGG-3') and Agro att reverse (5'-CGTGATGAGAGCGATACCG-3'). This DNA fragment was randomly labeled with [α-^{32}P]dCTP using the T7 Quick-Prime kit (Amersham Pharmacia). All probes were verified by restriction digests and/or sequencing of the cloned PCR products. Blots were exposed and quantitated using a Molecular Dynamics PhosphorImager.

Primer extension. The *ccrM* transcription start site was determined by primer extension analysis. Experiments were carried out with total RNA (20 to 40 μg) obtained from log-phase cultures of A136 and A348 using a standard hot phenol preparation method. The primer AgroMT17 (5'-GTCACTCTCGCCCGCACG-3') was 5' end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Boehringer) and separated from free nucleotide using the QIAquick nucleotide removal kit (Qiagen). Samples were annealed at 50° for 20 min following denaturation. Synthesis of cDNA was performed using SuperscriptII (Gibco-BRL) at 42°C for 1 h. The reactions were stopped, and the products were desalted on Spin-X columns (Costar) using Sephadex G25 resin (Amersham Pharmacia) and subjected to polyacrylamide gel electrophoresis in parallel with a sequencing ladder generated using the same primer and pLK300 as a template (Thermosequenase kit; Amersham Pharmacia).

Footprinting. DNase I protection experiments were performed as previously described with the purified *Caulobacter* CtrA response regulator (38). CtrA was phosphorylated using a maltose-binding protein–EnvZ fusion protein, also previously described (33). The template DNA was a 550-bp PCR product made using the primers AgMTFP1 (5'-CCGCACTTCAGGTTCCC-3') and AgMTFP2 (5'-CGTTGAGGATCCAGAAATCG-3'). The PCR product was 5' end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, and the 3' end of the labeled template was removed by digestion at a naturally occurring *Bam*HI site. A sequencing ladder generated with the AgMTFP1 primer was used to identify the protected region.

Synchronization of *A. tumefaciens* cultures. Cultures of *A. tumefaciens* were synchronized using a modification of the method described by Evinger and Agabian (10). Strain A348 was grown in LB at 28°C to an A_{600} of approximately 0.35. This culture was mixed with the colloidal silica preparation Ludox (420840; Aldrich) which had been brought to neutral pH by the addition of 1 N HCl immediately before use. The final ratio was 1.8 volumes of culture to each volume of neutralized Ludox. It should be noted that this ratio was empirically determined and may vary somewhat between batches of Ludox, that outside an appropriate range of ratios the cells all either stay at the top or go to the bottom, that the viscosity of Ludox changes over time after it has been neutralized, and that using a denser culture resulted in more contamination of the small cells with larger cells. The mixture was centrifuged in 15-ml Corex tubes (Corning) in a JA-20 rotor at 9,000 rpm (i.e., 9,800 × g) for 5 min at 4°C. Small, motile cells were found at the very bottom of the gradient, just above the clear liquid at the bottom, while most of the larger cells floated on top. Cells of intermediate size could be found in between. The small, motile cells from the bottom of the tube were collected, pooled, visually examined under a light microscope, and quickly washed two or three times in cold 1× AB without glucose, with pelleting at 9,000 rpm for 2 min each time. After washing, the pellet was resuspended in LB at 28°C, and the culture was incubated at 28°C. Samples were withdrawn at the indicated times, examined by light microscopy, and immediately either fixed as described above or frozen as pellets for further processing. During a cell cycle experiment, equal volumes of cells were withdrawn at each time point. For Western blotting, equal volumes of the samples were loaded on each lane of a protein gel.

Western blotting of flagellins. To examine flagellar protein levels by Western blotting, cell pellets from either mixed log-phase cultures or synchronous cultures were resuspended in 1× sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and run on an SDS–12% PAGE protein gel (35). These were transferred onto Immobilon P membranes (Millipore) using standard Western blotting techniques. Blots were probed with primary rabbit antiserum (raised to pooled *Caulobacter* flagellins) at a dilution of 1:5,000 (39),

followed by secondary antibody (donkey anti-rabbit immunoglobulin G–horseradish peroxidase conjugate; Jackson Laboratories) at a dilution of 1:10,000. Bound antibody was visualized by chemiluminescence. Autoradiograms were scanned using an Expression 800 scanner and Epson TWAIN Pro software (Epson America, Inc.).

Nucleotide sequence accession number. The sequence of the *A. tumefaciens ccrM* homolog has been submitted to GenBank under the accession number AF327563.

RESULTS

The *A. tumefaciens ccrM* gene is essential for viability. The full-length *A. tumefaciens ccrM* homolog was isolated from a genomic DNA library and sequenced as described in Materials and Methods. The encoded protein has 62% identity and 70% similarity to *Caulobacter* CcrM. While some of this homology is due to the conserved *S*-adenosylmethionine-binding and catalytic domains found in DNA methyltransferases, the C-terminal third of the protein is unique to the CcrM homologs. Compared to *Caulobacter* CcrM, the *S. meliloti* and *B. abortus* homologs have an N-terminal extension of 15 amino acids (45), while *A. tumefaciens* CcrM has an N-terminal extension of 20 amino acids (Fig. 1A). There are no open reading frames within 200 bp on either side of *A. tumefaciens ccrM*; a closely-linked open reading frame encoding a cognate restriction enzyme would be expected for a restriction-modification system (43). Thus, it is likely that, as in *Caulobacter*, *ccrM* is an orphan methyltransferase.

To determine whether *ccrM* is essential in *A. tumefaciens*, we replaced the sequence encoding the catalytic domain of the methyltransferase with a spectinomycin resistance cassette as described in Materials and Methods (Fig. 1B). This construct, on a vector which does not replicate in *A. tumefaciens* (pLK311), was transformed into strain A348. Integrants were isolated by selecting for the acquisition of kanamycin and spectinomycin resistance and then screened by Southern blotting. Strains LS3307 and LS3308 carry pLK311 integrated by recombination on the 5' and 3' sides of the spectinomycin resistance cassette, respectively. The low-copy-number vector pRK290(20R) alone or one containing *ccrM* was mated into LS3307 and LS3308, with subsequent selection on spectinomycin, kanamycin, and tetracycline. The resulting parent strains were grown without selection for either spectinomycin or kanamycin and then plated on minimal media containing 5% sucrose to select for a second recombination leading to excision of the *sacB* gene and vector. Isolates were then analyzed for spectinomycin and kanamycin resistance.

When no plasmid or the pRK290(20R) vector alone was present, recombination yielded only isolates sensitive to both kanamycin and spectinomycin, which therefore bore wild-type *ccrM* on the chromosome. However, if pLK308 was present, providing wild-type *ccrM* in *trans*, isolates bearing the disrupted copy of *ccrM* on the chromosome (spectinomycin resistant but kanamycin sensitive) could easily be obtained. Thus, *ccrM* is essential for viability in *A. tumefaciens*.

Overexpression of *ccrM* results in excess methylation of chromosomal DNA. CcrM overexpression has been associated with cell division defects and aberrant DNA replication in other alpha proteobacteria (34, 45, 48). To evaluate the effects of *ccrM* overexpression in *Agrobacterium*, the gene and its upstream region were cloned into the low-copy-number vector

pRK290(20R) (two to five copies per cell) and the high-copy-number vector pJS71 (20 to 30 copies per cell) (38). The CcrM homolog could not be detected, using anti-*Caulobacter* CcrM antibodies, in *A. tumefaciens* with a vector control or a low-copy-number plasmid bearing *ccrM*. However, in the strain with a high-copy-number plasmid bearing *ccrM*, a band of the appropriate size was detected, suggesting that a larger amount of CcrM protein was present (data not shown). To assess how this affected the methylation of genomic DNA, we used Southern blots to assay the methylation state of overlapping *Hinc*II-CcrM recognition sequences in the chromosomal *att* locus (Fig. 2A). The methylation state of the DNA determines its sensitivity to digestion at the overlapping *Hinc*II site; DNA which is fully methylated by CcrM cannot be cleaved by *Hinc*II. Passage of a replication fork through this fully methylated site yields two hemimethylated DNA products, only one of which can be cleaved by *Hinc*II.

For Fig. 2B, genomic DNA was digested with *Hinc*II. The top band contains fully methylated DNA and one hemimethylated replicative product; the lower band contains the other hemimethylated replicative product. In the wild-type strain, strains containing vector alone, and the strain carrying *ccrM* on a low-copy-number vector, there were similar ratios of counts in the lower band to counts in the upper band. However, when *ccrM* was present on a high-copy-number vector (pLK309), there was an 80 to 90% reduction in the amount of hemimethylated DNA, suggesting that following chromosome replication, the new DNA was rapidly brought to the fully methylated state. Southern blot assays of an overlapping *Cla*I-CcrM site in the chromosomal flagellar locus (9) confirmed this observation (data not shown). Therefore, when *ccrM* is present on a high-copy-number plasmid, GANTC sites are fully methylated much more often than under normal conditions.

Fully methylated chromosomal DNA is associated with morphological and flow-cytometric abnormalities. Microscopy (Fig. 2C) and flow cytometry analysis (Fig. 2D) of the strains bearing the plasmids referred to in Fig. 2B revealed that those carrying vector alone or *ccrM* on a low-copy-number plasmid had a normal cell size with unilateral outgrowth of progeny cells, and flow cytometry showed two discrete peaks of DNA content. These peaks correspond to one and two genome equivalents, respectively, as confirmed by flow cytometry of separated cell populations (described below; also, see Fig. 4). One genome equivalent is likely to represent the circular chromosome, linear chromosome, cryptic plasmid, and Ti plasmid of *A. tumefaciens* A348 (12). The strain in which *ccrM* is present on a high-copy-number plasmid has grossly abnormal morphology, with branching, elongated cells (Fig. 2C). The corresponding flow cytometry demonstrates a significantly increased DNA content (Fig. 2D). In an effort to determine whether there was selective alteration of chromosomal versus Ti plasmid replication, Southern blots of total DNA in this strain were probed for both the Ti plasmid and the chromosome. However, the ratio between the two was the same as observed in the wild-type, low-copy-number *ccrM*, and plasmid controls (data not shown).

The *A. tumefaciens* ccrM homolog has a conserved promoter structure and CtrA binding site. The start site of the *ccrM* transcript was mapped using primer extension and is shown in Fig. 3A and B. A strong signal was found 33 to 34 bases

upstream from the putative translational start site, which is preceded by a good consensus ribosome binding site. The same result was obtained with a different primer as well as with RNA from *A. tumefaciens* A136, which is isogenic to A348 but lacks the Ti plasmid (data not shown). Three different primers located upstream from the start site did not give a signal. Sequence comparison revealed extensive homology to the promoters of the *B. abortus* and *C. crescentus ccrM* homologs. At the −35 position relative to the transcriptional start site, there was a conserved binding motif for the CtrA response regulator (Fig. 3A); this binding motif has been extensively characterized in *Caulobacter* (28).

Although *ctrA* has not been cloned from *Agrobacterium*, a homolog of *Caulobacter ctrA* has been detected on Southern blots. CtrA is highly conserved in the other alpha proteobacteria from which it has been isolated (1, 19). A DNase I protection assay, described in Materials and Methods, was used to determine if the *Caulobacter* CtrA protein was able to bind to the conserved motif in the promoter region. Purified CtrA was phosphorylated in vitro using the *E. coli* histidine kinase EnvZ; this was verified in a parallel experiment using [γ-^{32}P]ATP. CtrA was able to protect a 26-bp region from −17 to −42 relative to the transcriptional start site; the presence of the protection pattern required phosphorylation of CtrA (Fig. 3C).

Methylation of the *A. tumefaciens* chromosome varies as a function of the cell cycle. A density centrifugation technique initially developed to synchronize cultures of *Caulobacter* (10) was used to separate small, motile cells from larger, mostly nonmotile cells of *A. tumefaciens*. As shown in Fig. 4A, fluorescence-activated cell sorter (FACS) analysis of these two populations showed that the small, motile cells from the bottom of the tube had a DNA content representing the 1N DNA fluorescence peak observed in a mixed cell population (Fig. 4A). The cells at the top had a DNA content superimposable on the 2N peak. We took the small, motile cells from the bottom of the tube and monitored their growth (Fig. 4B). These cells became nonmotile and began to elongate; they became motile again after they had doubled in size, and they divided at approximately 75 min following their initial resuspension in media. This was consistent with the growth rate of a mixed culture which did not undergo the separation process. As determined by FACS analysis, the DNA content gradually shifted from the 1N peak at the initial time point towards the 2N peak by 45 min. Reappearance of the 1N peak was associated with cell division and the reappearance of small motile cells in the culture, which was first observed at 60 min and was maximal at 75 min. At 90 min, the small cells had again begun to elongate and the DNA content to subtly increase. Thus, we successfully synchronized *Agrobacterium* cultures, allowing studies of the cell cycle.

We examined flagellin expression during the cell cycle to determine if it correlated with the variation in motility. Antibody to *Caulobacter* flagellins detected a protein(s) consistent with the size of *A. tumefaciens* flagellins (32 to 33 kDa); this band was absent in the NT1REB strain, from which the *flaA*, *flaB*, and *flaC* genes are deleted (8) (Fig. 4C). Western blots of samples taken from synchronized cultures showed that the expression of *A. tumefaciens* flagellar proteins is cell cycle dependent and maximal at times when the cells are motile.

The methylation state of the DNA at the *att* locus was as-

A.

HincII   HincII/
              CcrM   HincII

HincII   1.0 kb   0.3 kb
probe ▬▬▬▬

fully methylated DNA   HincII
not cleaved by HincII   — G T T G A^Me G T C —
1.3 kb   — C A A C T G A^Me G —

DNA replication

HincII   HincII
— G T T G A^Me T C —   — G T T G A C T C —
— C A A C T G A G —   — C A A C T G A^Me G —
hemimethylated DNA   hemimethylated DNA
not cleaved by HincII   cleaved by HincII
1.3 kb   1 kb

B.

┌ low copy ┐ ┌ high copy ┐

no plasmid
vector alone
vector + ccrM
vector alone
vector + ccrM

1.3 kb
FM + HM —
1 kb, HM —

ratio of bottom band to top band

C.

low copy vector   low copy vector   high copy vector   high copy vector
alone   + ccrM   alone   + ccrM

D.

cells

1N 2N   1N 2N   1N 2N

DNA fluorescence   DNA fluorescence   DNA fluorescence   DNA fluorescence
low copy vector   low copy vector   high copy vector   high copy vector
alone   + ccrM   alone   + ccrM

FIG. 2. Overexpression of the *ccrM* homolog leads to an increase in the methylation of chromosomal DNA. (A) Schematic of the DNA methylation assay. CcrM methylates adenine in the sequence GANTC (shaded). This CcrM site overlaps the *Hinc*II restriction enzyme recognition site GTTGAC in the *att* locus. If the DNA is fully methylated by CcrM, *Hinc*II will not be able to cleave it. However, once this DNA is replicated, each replication product will be hemimethylated, as shown below. The top strand is methylated in one product (left); the bottom strand is methylated in the other product (right). Only one of these two replication products can be cleaved by *Hinc*II, as shown. Southern blotting with the probe shown will yield a 1.3-kb band or a 1-kb band, depending on the methylation state of the DNA. (B) Methylation state of the *A. tumefaciens* A348 DNA in strains carrying different plasmids, using the differential *Hinc*II cleavage assay described for panel A. Genomic DNA from mixed cultures in exponential-phase growth was digested with *Hinc*II. The low-copy-number vector is pRK290(20R); the low-copy-number vector with *ccrM* is pLK308 [pRK290 (20R) with *ccrM*]; the high-copy-number vector is pJS71; the high-copy-number vector with *ccrM* is pLK309 (pJS71 with *ccrM*). The upper band contains fully methylated (FM) DNA and the hemimethylated (HM) DNA diagrammed on the left in panel A, while the lower band contains the hemimethylated DNA diagrammed on the right. Shown directly below is the ratio between bands for each lane of the gel. (C) Light microscopy of *A. tumefaciens* A348 carrying different plasmids, which are detailed for panel B above. Bar, 5 μm. (D) Flow cytometry of the strains in panel C. The *y* axis represents the number of cells with a given fluorescence intensity. The *x* axis shows the fluorescence intensity, which corresponds to DNA content.

A.

                               -35   **-10**

Ba   TTACGATTTCAAGCACTTGGCG**TTAA**CGGCATA**TTTACCC**TACGCAGTAACCATAGGAAC $^{+1}$

At   ATTTCGCATTTTCCGTGA**CTT...GTTCAGGCTTTGATAAGCATCTT**CGGTAACCATAAGCGTC

Cc   AAGCGCCTGAAAGGCCGTG...**GTTAA**CGGCCCGC**TAACC**ACGTCTCTCAACACCGGATTTAC

B.

C.
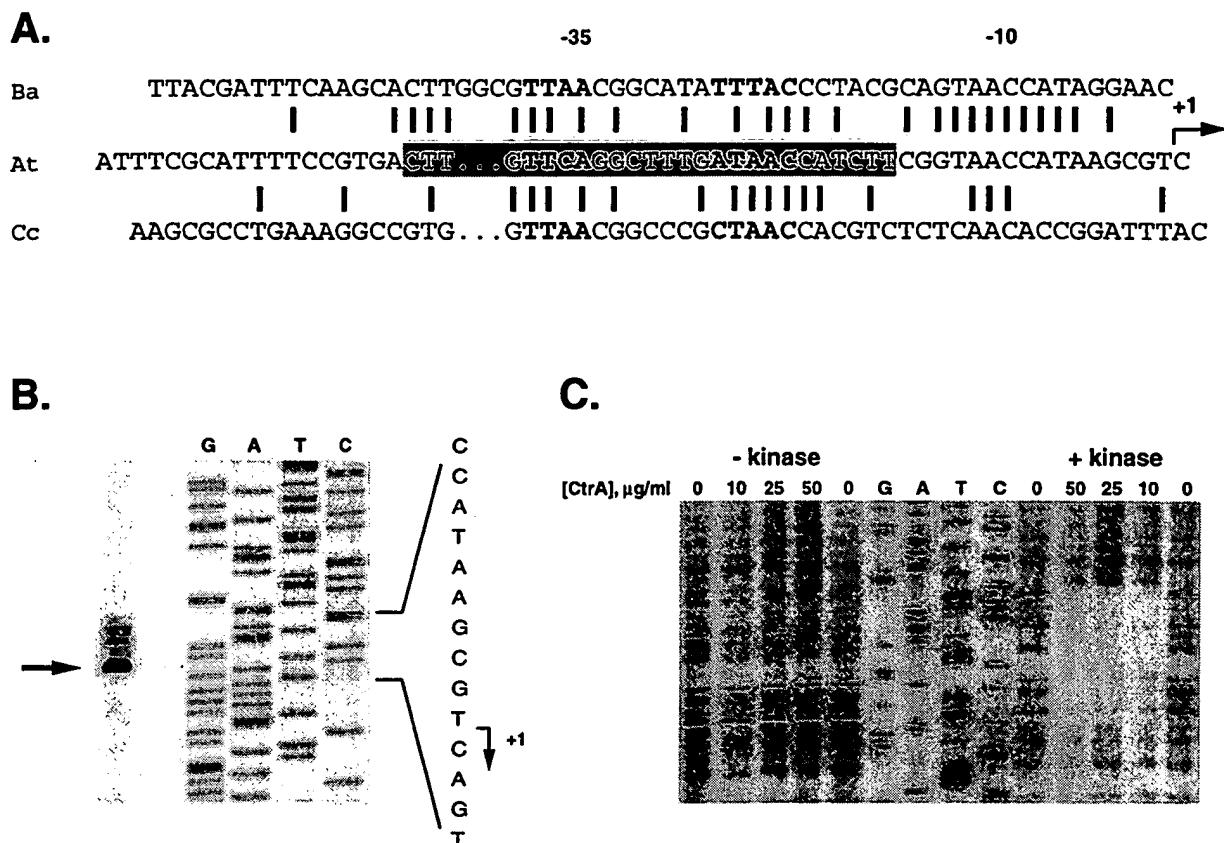
FIG. 3. The *A. tumefaciens* *ccrM* promoter has a highly conserved sequence and is bound by the CtrA response regulator. (A) Sequence of the *ccrM* promoter region and its alignment with the *ccrM* promoters of *C. crescentus* and *B. abortus*. The conserved CtrA binding site (consensus, TTAANTTAAC) is in bold type. The region protected from DNase I by phosphorylated CtrA is shaded. (B) Start site of *ccrM* transcription as determined by primer extension. A sequence ladder generated with the same primer was used to determine the start site. (C) DNase I protection of the *ccrM* promoter by CtrA. A sequence ladder generated by using the primer AgMTFP1 was used to identify the protected region. Protection was seen only if CtrA was phosphorylated by the histidine kinase EnvZ.

sessed by the restriction enzyme digestion assay as described in Fig. 2. DNA was prepared from samples taken from synchronized cultures. Figure 4D shows that the degree of DNA methylation at this locus varied over the course of the cell cycle: as DNA replication proceeded, the *att* locus became completely hemimethylated by 30 min. A ratio of one between the bottom and top band on the blot indicates 100% hemimethylation of the DNA. After the completion of replication at 45 min, the DNA became newly methylated and was maximally so at 60 and 75 min, when cell division occurred. As the cells began to replicate their DNA at 90 min, the amount of hemimethylated DNA increased again. Thus, the activity of the CcrM methyltransferase is restricted to late in S phase of the cell cycle of *A. tumefaciens*, prior to cell division.

DISCUSSION

We have characterized the *ccrM* homolog from *A. tumefaciens* and have shown that it is essential for viability. This requirement is unlikely to be due to restriction of DNA: the gene has no linked open reading frames as would be expected of a restriction-modification system (43). In *Caulobacter*, furthermore, *ccrM* has no adjacent open reading frames; promiscuously replicating plasmid DNA can be isolated which is un-

methylated at *Hin*fI sites, suggesting that there is in fact no restriction endonuclease with the same sequence specificity as CcrM (48). Like its homologs in *C. crescentus*, *S. meliloti*, and *B. abortus*, therefore, *A. tumefaciens* *ccrM* appears to be an essential orphan methyltransferase.

In addition to the requirement for CcrM methylation in all bacteria where it has been isolated thus far, its overexpression causes significant abnormalities of morphology and DNA content (34, 45). In *Agrobacterium*, branched, elongated cells with multiple chromosomes were observed when *ccrM* was present on a high-copy-number plasmid. In this strain, DNA was maintained in a fully methylated state. Thus, inappropriate DNA methylation by CcrM interferes with DNA replication and cell division, or the coordination between the two. In *E. coli*, temporally synchronized initiation at several origins of replication within each rapidly growing cell is dependent on the level of Dam methyltransferase and becomes uncoordinated when the level of Dam is either too high or too low (4). It has been established that the hemimethylated origin in *E. coli* is incompetent for replication initiation and that its physical sequestration and delayed methylation due to the SeqA system are important in ensuring synchronous timing of initiations (5). While the recently completed *Caulobacter* genome (26) does

A.
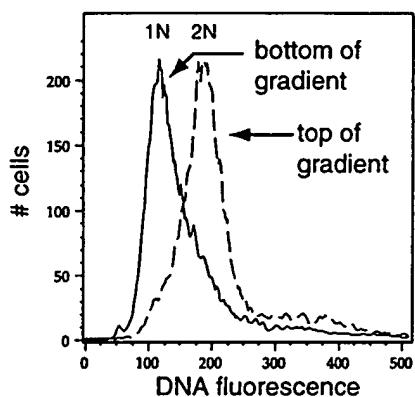
B.
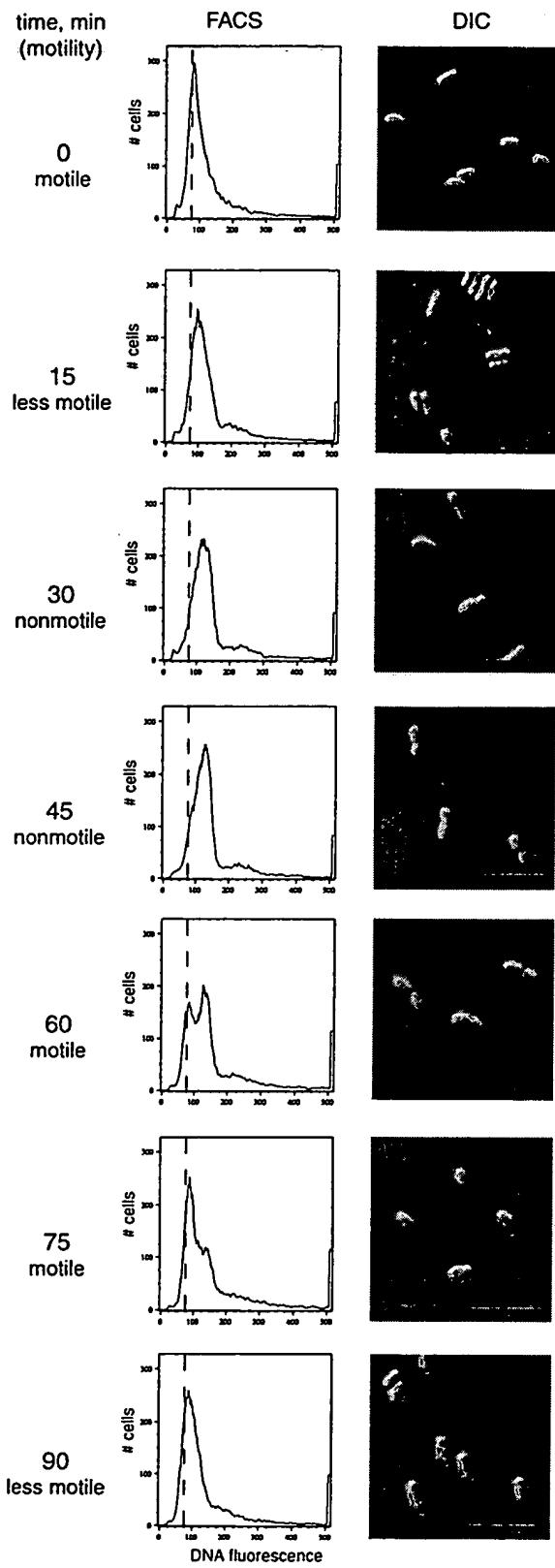
C.
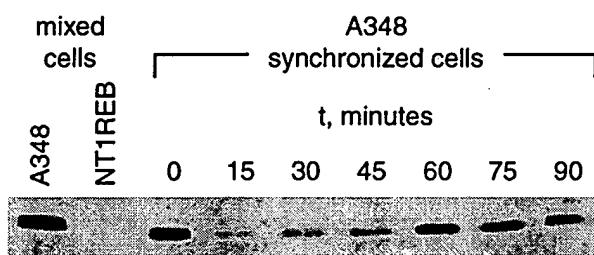
D.
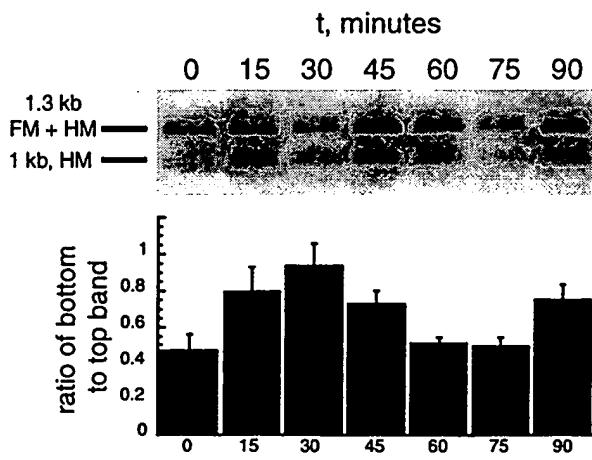
not contain a SeqA homolog, CcrM methylation sites are found in the *Caulobacter* origin of replication at a frequency higher than expected by random chance, and studies to address whether they are important in the initiation of DNA replication are ongoing. As discussed below, there may be some parallels between *Agrobacterium* and *Caulobacter* replication, although the *Agrobacterium* chromosomal origin of replication has not yet been characterized.

No difference from wild-type levels of methylation or any obvious morphological abnormality was seen when *ccrM* was present on a low-copy-number plasmid. This is probably due to dual regulation of CcrM levels, as in *Caulobacter*, where a more robust transcriptional increase is needed to overcome proteolysis. However, it is possible that low-level, yet temporally inappropriate, DNA methylation can affect other cellular processes which are more difficult to assay in *A. tumefaciens*. In support of this hypothesis is the observation that *ccrM* on a low-copy-number plasmid can, without causing morphologic abnormalities, cause attenuation of intracellular bacterial proliferation in a macrophage model of *B. abortus* infection (34). Thus, as in the enteric bacteria, methylation may regulate diverse cellular functions among members of the alpha subdivision.

It is possible that CcrM DNA methylation in *Agrobacterium* also affects transcription of a number of genes. In *Caulobacter*, CcrM exerts negative control on its own promoter, repressing transcription in the swarmer cell (40). The abnormal branching morphology we observed in *A. tumefaciens* or in *S. meliloti* (45) overexpressing CcrM is similar to that associated with blockage of the cell cycle in *S. meliloti* by treatment with either DNA-damaging agents like nalidixic acid and mitomycin C or inhibition of septation by cephalexin (20). Thus, in addition to any effects it may have on chromosomal replication, inappropriate DNA methylation in *Agrobacterium* may also up- or down-regulate genes involved in normal cell cycle progression.

We have mapped the transcriptional start site of *Agrobacterium ccrM* and found that its upstream region is highly homologous to the promoters of the *Caulobacter* and *Brucella ccrM* genes. A notable feature of this region is a conserved binding motif for the global response regulator CtrA. While the CtrA homolog has not yet been cloned from *Agrobacterium*, it has been found in several other alpha proteobacteria and is highly conserved, with ≥80% identity between *Caulobacter* CtrA and its *Sinorhizobium* and *Brucella* homologs (1). CtrA has been shown to be essential for viability in both *Caulobacter* and *S. meliloti* (1, 30). It positively regulates *ccrM* transcription in *Caulobacter*, and it footprints the *ccrM* promoter in both *Caulobacter* and *Brucella* (33, 34). We have demonstrated that purified *Caulobacter* CtrA footprints the region of the *A. tumefaciens ccrM* promoter containing the conserved CtrA binding motif, which overlaps the −35 position relative to the transcriptional start site. This interaction, as for known CtrA targets in *Caulobacter*, occurs in a phosphorylation-dependent manner (17, 31, 33, 38). Thus, it is likely that in *A. tumefaciens*, CtrA also regulates *ccrM* transcription. In conjunction with what is known about *Caulobacter* and *Brucella*, this suggests conservation among the alpha bacteria of a regulatory mechanism governing *ccrM* expression. Site-directed mutation of methylation sites in the 5' untranslated region of *Caulobacter ccrM* has been found to prolong its expression during the cell cycle, suggesting possible autoregulation (41). In contrast to the *Caulobacter* promoter, the promoters of the *A. tumefaciens* and *B. abortus ccrM* homologs do not have methylation sites. This may reflect a need for more stringent temporal modulation of CcrM expression in *Caulobacter*, perhaps due to its asymmetric cell division.

In this work, we demonstrate for the first time that it is feasible to observe the growth of synchronized cultures of *A. tumefaciens*, thus establishing it as another model organism for studying the bacterial cell cycle. We monitored DNA replication, cell division, DNA methylation state, motility, and flagellar protein expression over the course of the cell cycle. Cells doubled in length as they doubled their DNA content; this was followed by cell division and a concomitant reduction in ploidy. Because of innate drug resistance, we could not treat the cultures with antibiotics which would block initiation and allow us to count the number of origins per cell. However, we consistently observed only 1N and 2N peaks on flow cytometry of wild-type cells, rendering it likely that DNA replication in *Agrobacterium* occurs just once per cell cycle. DNA replication in *Caulobacter* has been shown to occur once and only once per cell cycle (22), in contrast to the multiple rounds which can be seen in *E. coli* (37). Our findings suggest that this precise control of DNA replication may be common to other alpha proteobacteria as well. We also observed that motility varied during the *Agrobacterium* cell cycle and correlated well with flagellin expression. Flagellation may facilitate virulence in *Agrobacterium* (8) and is downregulated in a strain-dependent manner under conditions inducing T-pilus biogenesis (18). In light of our work, motility also appears to be influenced by innate cell cycle cues, and studying temporal aspects of its regulation is now possible.

Finally, we demonstrate that methylation of the *A. tumefaciens* chromosome is constrained to a specific period of the cell cycle, as is the case in *Caulobacter*. In *E. coli*, it has been established that, with the exception of the origin of replication,

FIG. 4. *A. tumefaciens* A348 can be synchronized, and the methylation state of the chromosome varies as a function of the cell cycle. (A) Cells were separated using density centrifugation, as described in Materials and Methods. Flow cytometry of the cells isolated from the bottom and the top of the gradient is shown. (B) The cells from the bottom of the gradient were isolated and allowed to grow as described in Materials and Methods. The changes in motility are indicated; DNA content, as assessed by flow cytometry, and cell morphology over time are shown. The assessment of motility reflects the behavior of the majority of the cell population, as observed by light microscopy, at the time the sample was taken. A vertical line has been arbitrarily placed at 75 fluorescence units on the *x* axis to aid in following the relative positions of the peaks over the course of the experiment. Bar, 5 μm. (C) Western blot of *A. tumefaciens* flagellin proteins using polyclonal antiserum to *Caulobacter* flagellins. The specific 32- to 33-kDa band is shown. Mixed cell populations from both wild-type (A348) and flagellin-negative (NT1REB) cells are shown in the first and second lanes as positive and negative controls. The remaining lanes track the flagellins over the course of the cell cycle in A348. (D) Genomic DNA was prepared from cells at the time points shown in panel B, and the methylation state of the *att* locus was assayed as described in Materials and Methods and for Fig. 2. A ratio of one between the bottom band and the top band corresponds to 100% hemimethylation of the DNA.

the daughter chromosomes are swiftly methylated by Dam after the replication fork passes. Due to the rapidity of this process, hemimethylated DNA is difficult to detect at the sites outside the origin, and complete hemimethylation of the DNA is never attained (5). In contrast, it is clear that in *Caulobacter*, the restriction of CcrM to a narrow window of the cell cycle leads to prolonged hemimethylation of the chromosome during the cell cycle. Although we are currently unable to assess cell cycle dependence of *A. tumefaciens ccrM* transcription and CcrM protein levels, our DNA methylation assay evaluates CcrM activity directly. Our inability to knock out the gene makes it unlikely that there is functional redundancy of this methylating activity. We demonstrate here that the *att* locus becomes completely hemimethylated 15 min before the time at which all cells in the population have replicated their DNA, presumably due to passage of the replication fork. Methylation only occurs coincident with or immediately following completion of DNA replication, indicating that the presence and/or activity of *A. tumefaciens* CcrM is similarly temporally restricted.

In *Caulobacter*, the swarmer cells isolated at the beginning of a synchrony are unable to initiate replication. Hemimethylated DNA is detected only later in the cell cycle, as mature swarmer cells develop into replication-competent stalked cells. However, it is unclear whether *A. tumefaciens* is ever in an initiation-incompetent form. Immediate reinitiation could account for our observation that the *A. tumefaciens* DNA was not fully methylated at the beginning of the synchrony. It is also possible that we are unable to obtain starting cell populations as synchronized as in *C. crescentus*.

It has been established that CcrM is widespread in the alpha subdivision of bacteria (45). Here, we present evidence that temporally constrained DNA methylation activity may be a conserved means of regulating fundamental aspects of the cell cycle common to all these bacteria. Thus, CcrM appears to be an orphan methyltransferase which, like Dam, provides important regulatory information. The alpha proteobacteria include the plant and animal pathogens *Agrobacterium*, *Brucella*, and *Bartonella*; more distant CcrM homologs which are part of restriction-modification systems have been found in *H. influenzae* and *Helicobacter pylori*. Thus, inhibition of CcrM function may prove lethal to such bacteria, either through disruption of an essential regulatory function or by allowing digestion of unmethylated DNA to occur. Just as Dam has become a therapeutic target in bacteria, CcrM may be another attractive candidate target for the development of methyltransferase inhibitors as novel antibiotics.

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